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Generation of $^{\cdot}OH$ initiated by interaction of Fe^{2+} and Cu^{+} with dioxygen; comparison with the Fenton chemistry★✪

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Iron and copper toxicity has been presumed to involve the formation of hydroxyl radical ($^{\cdot}OH$) from H_2O_2 in the Fenton reaction. The aim of this study was to verify that Fe^{2+}-O_2 and Cu^{+}-O_2 chemistry is capable of generating $^{\cdot}OH$ in the quasi physiological environment of Krebs-Henseleit buffer (KH), and to compare the ability of the Fe^{2+}-O_2 system and of the Fenton system (Fe^{2+} + H_2O_2) to produce $^{\cdot}OH$. The addition of Fe^{2+} and Cu^{+} (0–20 μM) to KH resulted in a concentration-dependent increase in $^{\cdot}OH$ formation, as measured by the salicylate method. While Fe^{3+} and Cu^{2+} (0–20 μM) did not result in $^{\cdot}OH$ formation, these ions mediated significant $^{\cdot}OH$ production in the presence of a number of reducing agents. The $^{\cdot}OH$ yield from the reaction mediated by Fe^{2+} was increased by exogenous Fe^{3+} and Cu^{2+} and was prevented by the deoxygenation of the buffer and reduced by superoxide dismutase, catalase, and desferrioxamine. Addition of 1 μM, 5 μM or 10 μM Fe^{2+} to a range of H_2O_2 concentrations (the Fenton system) resulted in a H_2O_2-concentration-dependent rise in $^{\cdot}OH$ formation. For each Fe^{2+} concentration tested, the $^{\cdot}OH$ yield doubled when the ratio $[H_2O_2]$:$[Fe^{2+}]$ was raised from zero to one. In conclusion: (i) Fe^{2+}-O_2 and Cu^{+}-O_2 chemistry is capable of promoting $^{\cdot}OH$ generation in the environment of oxygenated

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Abbreviations: DFX, desferrioxamine; DHBAs, 2,5- and 2,3-dihydroxybenzoic acids; DTPA, diethylenetriaminepentaacetic acid; HPLC, high performance liquid chromatography; KH, Krebs-Henseleit buffer; SOD, superoxide dismutase.

KH, in the absence of pre-existing superoxide and/or H_2O_2, and possibly through a mechanism initiated by the metal autoxidation; (ii) The process is enhanced by contaminating Fe^{3+} and Cu^{2+}; (iii) In the presence of reducing agents also Fe^{3+} and Cu^{2+} promote the $^{\cdot}OH$ formation; (iv) Depending on the actual $[H_2O_2]$:$[Fe^{2+}]$ ratio, the efficiency of the Fe^{2+}-O_2 chemistry to generate $^{\cdot}OH$ is greater than or, at best, equal to that of the Fe^{2+}-driven Fenton reaction.

Oxygen derived oxidants, such as superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$), have been implicated as causative agents in various disease states and tissue injuries [1]. A long standing proposal is that $O_2^{\cdot-}$ is first generated in a variety of enzymatic reactions. Then it furnishes H_2O_2 (Eqn. 1) and also interacts with a transition metal, such as iron or copper, to regenerate its reduced form (Eqn. 2). Thereby $O_2^{\cdot-}$ provides substrates for the Fenton reaction which generates a powerful oxidant, $^{\cdot}OH$ (Eqn. 3).

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 \quad (1)$$

$$O_2^{\cdot-} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (2)$$

$$H_2O_2 + Fe^{2+} \rightarrow {}^{\cdot}OH + OH^- + Fe^{3+} \quad (3)$$

However, an alternative mechanism would be that biological free radical oxidations are a consequence of a direct interaction between a metal ion and dioxygen (O_2). Arguments in favor of this concept are that: (i) in biological systems, the concentration of O_2 is usually much greater than that of H_2O_2; (ii) the rate constants for the Fe^{2+}-O_2 and Fe^{2+}-H_2O_2 reactions are similar ($10^3\ M^{-1}\ s^{-1}$), and (iii) an unbound catalytic metal may be present mostly in its reduced form [2, 3], thereby favoring its direct interaction with O_2 rather than with H_2O_2 (see ref. [3] for an extensive review). In this context, it has been proposed that it is iron-oxygen complexes, like perferryl and ferryl ions, which are responsible for iron-induced oxidation of various biochemical targets [3–5]. On the other hand, it has been demonstrated that a direct reduction of O_2 by Fe^{2+} (Eqn. 4), the process referred to as the metal autoxidation reaction, may mediate $^{\cdot}OH$ generation [6–9] (Eqn. 4).

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot-} \quad (4)$$

The dismutation of $O_2^{\cdot-}$ to H_2O_2 and the reaction of the latter with the remaining Fe^{2+} would eventually promote the liberation of $^{\cdot}OH$ in a fashion similar to that summarised in reactions (1) and (3). Nevertheless, the biological significance, if any, of Fe^{2+}-O_2 and/or Cu^+-O_2 chemistry-mediated $^{\cdot}OH$ generation is not known, particularly that the process has been studied mostly in the presence of a nonphysiological metal chelator (e.g., EGTA) [3, 6–9].

The aim of the present study was to verify that Fe^{2+}-O_2 and Cu^+-O_2 chemistry is capable of generating $^{\cdot}OH$ in Krebs-Henseleit buffer (KH), a medium routinely used in physiological studies, and to compare the ability of the Fe^{2+}-O_2 system and the Fenton system (Fe^{2+} + H_2O_2) to produce $^{\cdot}OH$. To verify that the metal ion-O_2 chemistry results in the autoxidative $^{\cdot}OH$ formation, the involvement of O_2, $O_2^{\cdot-}$ and H_2O_2 in the reaction was studied. It was also verified that, in the presence of a reductant, ferric and cupric ions are capable of mediating $^{\cdot}OH$ production in the absence of pre-existing $O_2^{\cdot-}$ and/or H_2O_2. The study demonstrates that, at least in the environment of highly oxygenated KH, the efficiency of the Fe^{2+}-O_2 system to generate $^{\cdot}OH$ is greater than or, at best, equal to that of the Fe^{2+}-driven Fenton reaction.

MATERIALS AND METHODS

Reagents and solutions. Ascorbic acid, catalase (bovine liver), desferrioxamine mesylate (DFX), diethylenetriaminepentaacetic acid (DTPA), 2,3-dihydroxybenzoic acid (2,3-DHBA), 2,5-dihydroxybenzoic acid (2,5-DHBA), $FeSO_4 \cdot 7H_2O$, glutathione (reduced form), D,L-homocysteine, sodium salicylate, NaCl, $NaHCO_3$, KCl and $CaCl_2 \cdot 2H_2O$ were purchased from Sigma (St. Louis, MO, U.S.A.). $FeCl_3 \cdot 6H_2O$, CuCl, $CuCl_2 \cdot 2H_2O$, L-cysteine, KH_2PO_4 and $MgSO_4$ were from Merck (Darmstadt, Germany), superoxide dismutase (bovine erythrocytes, SOD) from Boehringer Mannheim and H_2O_2 from Chempur (Gliwice, Poland).

All reagents were of the highest available purity and were used without further purification. All iron and copper ion solutions were prepared fresh as concentrated stock solutions in deionised (Milli-Q, Millipore-Waters, U.S.A.) and deaerated H_2O purged with nitrogen and were used immediately. Under these circumstances, precipitation of Fe^{3+} as $Fe(OH)_3$ was not a problem. Cuprous ion solutions were prepared by dissolving CuCl directly in acidified KH (see later). The glassware containing Fe^{2+} and Cu^+ was protected from light. H_2O_2 stock solutions were prepared from 30% parent solution and regularly titrated against $KMnO_4$. When needed, catalase and SOD were inactivated by heating their concentrated solution at 45°C and 90°C, respectively, for 20 min.

All experiments were performed at room temperature, and if not otherwise stated, in KH containing (in mM): NaCl 118; $NaHCO_3$ 23.8; KCl 4.7; KH_2PO_4 1.2; $CaCl_2$ 2.5; $MgSO_4$ 1.2. The buffer was supplemented with 1 mM sodium salicylate, which served as a specific trap for ·OH [10]. The buffer was equilibrated with a 95% O_2 + 5% CO_2 gas mixture giving pH 7.4 and pO_2 590–630 mmHg ($[O_2]$ about 1000 μM). In some experiments pH of the buffer was reduced to 6.6 by decreasing $NaHCO_3$ to 3 mM. To deoxygenate the buffer, it was equilibrated with a 95% N_2 + 5% CO_2 gas mixture giving pH 7.4 and pO_2 5–9 mmHg ($[O_2]$ about 10 μM).

Iron ion-mediated ·OH generation. The following reagents were mixed in the order stated:

- 0.8 ml KH buffer (1.25 times concentrated)
- 0.1 ml distilled water
- 0.1 ml $FeSO_4$ or $FeCl_3$ (0–20 μM)

When the effect of SOD, catalase, DFX, DTPA, Fe^{3+} and Cu^{2+} on the Fe^{2+}-mediated ·OH production was examined, 0.1 ml of the studied agent was added instead of 0.1 ml distilled water and the reaction was initiated by adding 0.1 ml of 100 μM Fe^{2+} solution (final concentration 10 μM). In the majority of the experiments the reaction mixture was incubated for 60 s. Then a 20 μl aliquot was injected into HPLC to detect 2,5- and 2,3-dihydroxybenzoic acids (DHBAs) formed. Preliminary experiments revealed no change in the ·OH yield with the incubation times varying from 30 s up to 30 min (Fig. 1).

Salicylate is known to chelate iron [6] and therefore some of the chemistry studied here may be due to radical formation by this complex rather than by a complex with, for instance, phosphate contained in KH. To check for this, the Fe^{2+}-mediated ·OH generation was studied also in KH devoid of phosphate (phosphate-free KH).

Copper ion-mediated ·OH production. Because of the poor water solubility of CuCl, its solutions were prepared by dissolving CuCl (0–20 μM) directly in acidified KH ($NaHCO_3$ reduced to 3.0 mM to give pH 6.6) containing salicylate and in some experiments 10 μM DFX. For comparison, also the Fe^{2+} autoxidation reaction was studied in acidified KH. The reaction mixtures containing copper ions were incubated for 30 min before the HPLC measurements were performed.

Iron ion- and copper ion-mediated ·OH formation in the presence of reducing agents. $FeSO_4$, $FeCl_3$ or $CuCl_2$ was added to the solution of ascorbic acid, reduced glutathione, cysteine or homocysteine in KH.

Usually the reaction mixture was incubated for 5 min. In the ascorbate experiments, the DHBAs generation was followed for 90 min. The final concentration of the metal was 10 μM and that of the reductor 200 μM.

·OH generation in the Fe^{2+}-driven Fenton reaction. $FeSO_4$ (1 μM, 5 μM or 10 μM) was added to oxygenated KH containing increasing concentrations of H_2O_2 (0–35 μM). The reaction mixtures were incubated for 60 s.

Analytical procedures. Hydroxyl radical generation was determined by measuring the formation of dihydroxybenzoic acid isomers (DHBA) from salicylate. 2,5-DHBA and 2,3-DHBA were separated by HPLC and quantified by an electrochemical detector as described by Floyd *et al.* [10]. HPLC measurements were performed on a Shimadzu System (Kyoto, Japan) consisting of a LC-6A Solvent Delivery Pump, a L-ECD-6A Electrochemical Detector, a SCL-6B Controller, a Chromatopac C-R4A data processor and an on-line ERC-3312 Degasser (Erma, Tokyo, Japan). A Macherey-Nagel Nucleosil C_{18} 250 × 4.6 mm reverse phase column was used for the separation (Duren, Germany). The eluent was 96% (v/v) 45 mM citrate/61 mM sodium acetate/47 mM acetic acid buffer (pH 3.6)/4% methanol. It was filtered through a 0.22 μm membrane filter (Millipore, Ireland) and pumped at a flow rate of 1.5 ml/min.

The reaction mixture pO_2 was measured with a Ciba-Corning 248 pH/Gas Analyser (Essex, England).

Statistics. All data are expressed as mean ± S.E.M. Significant differences ($P < 0.05$) among groups were calculated by one-way analysis of variance followed by the Dunnet's procedure. Non-paired Student's *t*-test was also used when appropriate.

RESULTS

Fe^{2+}- and Cu^{+}-mediated production of ·OH

Some 2,5- and 2,3-DHBA was present already in the oxygenated, salicylate containing phosphate-free KH as well as standard KH (Table 1). The addition of Fe^{2+} to these solutions caused a further rise in DHBAs forma-

Table 1. Dihydroxybenzoic acids yield from the reaction mediated by Fe^{2+} and Cu^{+}, as affected by superoxide dismutase, catalase, DTPA and desferrioxamine

Reaction mixture	n	Dihydroxybenzoic acids (pmol/ml)		
		2,5-DHBA	2,3-DHBA	Total
Phosphate-free KH	5	0.4±0.1*	0.4±0.1*	0.8±0.1*
+ Fe^{2+}, 10 μM	5	3.0±0.2*	1.8±0.2*	4.9±0.3*
KH buffer	10	0.4±0.1*	0.5±0.1*	1.0±0.1*
+ Fe^{2+}, 10 μM	37	11.5±0.4	10.0±0.4	21.6±0.5
+ Fe^{2+} + Superoxide dismutase, 500 U/ml	6	8.8±0.8*	5.9±0.5*	14.6±1.2*
+ Fe^{2+} + Superoxide dismutase, boiled	5	14.0±1.6	10.4±1.0	24.3±2.9
+ Fe^{2+} + Catalase, 1000 U/ml	6	8.7±0.7*	7.3±0.4*	16.0±0.9*
+ Fe^{2+} + Catalase, boiled	6	18.7±1.2*	13.2±0.4*	31.9±1.5*
+ Fe^{2+} + DTPA, 10 μM	6	1.4±0.3*	1.3±0.4*	2.7±0.6*
+ Fe^{2+} + Desferrioxamine, 10 μM	11	2.1±0.4*#	2.4±0.4*#	4.5±0.6*#
+ Fe^{2+} + Desferrioxamine, 50 μM	5	2.9±0.6*#	1.4±0.3*	4.3±0.3*#
+ Cu^{+}, 10 μM	5	14.3±1.0	12.0±0.6	26.3±1.7
+ Cu^{+} + Desferrioxamine, 10 μM	5	3.7±0.6*	3.1±0.4*	6.8±0.7*

The values represent the mean ± S.E.M. of n determinations. The reaction mixtures containing Fe^{2+} were incubated for 60 s at room temperature in oxygenated phosphate-free or phosphate-containing Krebs-Henseleit buffer at pH 7.4, each containing 1 mM sodium salicylate. The reaction mixtures containing Cu^{+} were incubated at pH 6.6 for 30 min. An aliquot was then injected into HPLC to detect 2,5- and 2,3-dihydroxybenzoic acids (DHBAs) formed. DTPA, diethylenetriaminepentaacetic acid; *$P < 0.05$ *vs* KH buffer + Fe^{2+} alone or *vs* KH buffer + Cu^{+} alone, respectively #$P < 0.05$, DTPA *vs* desferrioxamine.

tion, which was, however, 4.5-fold greater in the presence of phosphate (Table 1).

The maximum level of DHBAs was reached already after 30 s of Fe^{2+} incubation in KH. Deoxygenation of the reaction mixture prior to the addition of Fe^{2+} prevented DHBAs formation (Fig. 1).

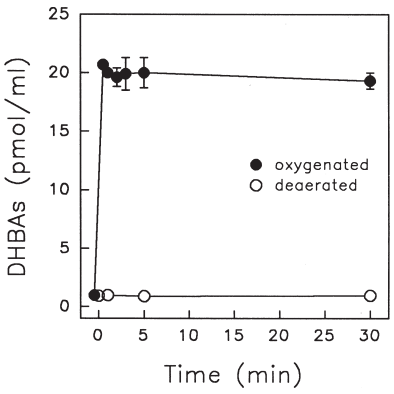

Figure 1. Time-course of Fe^{2+}-induced formation of 2,5- plus 2,3-dihydroxybenzoic acids (DHBAs).

At time 0, 10 μM Fe^{2+} was added to the Krebs-Henseleit buffer containing 1 mM salicylate and the reaction was followed for 30 min. The buffer was pre-equilibrated either with 95% O_2 + 5% CO_2 (oxygenated, pO_2 614±15 mmHg) or with 95% N_2 + 5% CO_2 gas mixture (deoxygenated, pO_2 6.8 ± 1.5 mmHg). The values represent the mean ± S.E.M. of 4–5 determinations.

The ·OH formation increased with the concentration of Fe^{2+} or Cu^{+} (0–20 μM) in the medium (Fig. 2). Approximately three times more ·OH was formed by Cu^{+} than by an equimolar concentration of Fe^{2+}, at least as evidenced by the measurements performed at pH 6.6. In addition, the reaction showed a pH dependency, as evidenced by the fact that Fe^{2+}-mediated ·OH production was approximately doubled when the pH of the reaction mixture was increased from 6.6 to 7.4. The addition of a range of Fe^{3+} or Cu^{2+} concentrations to KH did not result in ·OH formation (Fig. 2).

The ·OH formation mediated by 10 μM Fe^{2+} was reduced by 32% and 26% by SOD (500 U/ml) and catalase (1000 U/ml), respectively, and it was not affected and augmented by heat-inactivated SOD and catalase, respectively (Table 1).

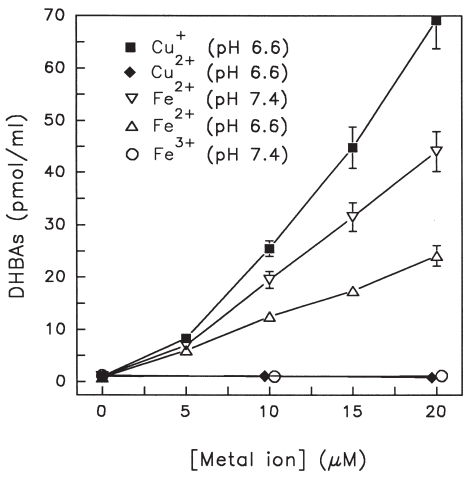

Figure 2. The concentration-dependency of copper ion- and iron ion-mediated formation of 2,5- plus 2,3-dihydroxybenzoic acids (DHBAs).

The reaction mixture containing the indicated concentration of iron and copper ions was incubated for 1 min and 30 min, respectively, in oxygenated Krebs-Henseleit buffer. The values represent the mean ± S.E.M. of 5–37 determinations.

The role of Fe^{3+} and Cu^{2+} in the Fe^{2+}- and Cu^{+}-mediated ·OH formation

The nonspecific Fe^{2+}/Fe^{3+} chelator DTPA (10 μM) and the specific Fe^{3+} chelator DFX (10 μM) reduced ·OH yield in the reaction mediated by 10 μM Fe^{2+} by 88% and 79%, respectively ($P < 0.05$, Table 1). ·OH generation was not attenuated further even when 50 μM DFX was used (Table 1). These results imply that the chelation of Fe^{3+} was mainly responsible for the effect of DTPA and DFX, and that Fe^{2+}-mediated reaction was strongly dependent on the contaminating Fe^{3+}. To examine this, the reaction mediated by 10 μM Fe^{2+} was studied in the presence of extra Fe^{3+} added to the reaction mixture. As demonstrated in Fig. 3, the Fe^{3+} concentration-dependent rise in the ·OH production was observed. The process saturated at the Fe^{3+} : Fe^{2+} concentration ratio of about 10, resulting in a 60%, at the maxi-

mum, rise in the ·OH generation. However, in our experiments, only media containing DFX may be regarded as devoid of the contaminating Fe^{3+}. If this is taken into account, it appears that ·OH yield from the Fe^{2+}-mediated reaction increases as much as ten-fold when the contaminating Fe^{3+} concentration increases from zero (DFX added to the medium) to that resulting in saturation (Fig. 3).

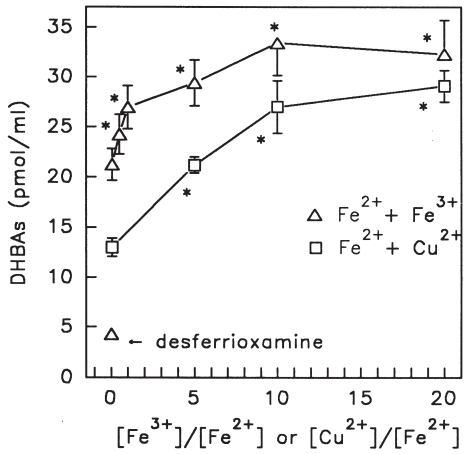

Figure 3. Effect of Fe^{3+} (Δ) and Cu^{2+} (£) on Fe^{2+}-mediated formation of 2,5- plus 2,3-dihydroxybenzoic acids (DHBAs).

Oxygenated KH contained 0, 5, 10, 50, 100 or 200 μM Fe^{3+} (at pH 7.4) or 0, 50, 100 or 200 μM Cu^{2+} (at pH 6.6) and the reaction was started by the addition of 10 μM Fe^{2+}. The arrow points to the DHBAs production in the reaction mixture containing 10 μM desferrioxamine and 10 μM Fe^{2+} (data repeated from Table 1). The values represent the mean ± S.E.M. of 5–7 determinations. *$P < 0.05$ *vs* zero Fe^{3+} or zero Cu^{2+}.

The Fe^{2+}-mediated ·OH yield was enhanced not only by Fe^{3+} but also by Cu^{2+} (Fig. 3). Likewise, DFX reduced the ·OH yield from the Cu^{+}-mediated reaction (Table 1).

Iron- and copper-mediated ·OH formation is enhanced by reducing agents. Ascorbate at 200 μM, added to KH alone or to the reaction mixture containing 10 μM Fe^{3+} or Fe^{2+}, resulted in a steady ·OH formation during the whole 90 min observation period (Fig. 4). However, the rate of ·OH formation was greatly increased in the medium containing Fe^{3+} or Fe^{2+}, compared with that in KH alone. DFX not only lowered the ·OH yield, but also completely prevented its time-dependent rise in the medium containing Fe^{2+} and ascorbate (Fig. 4).

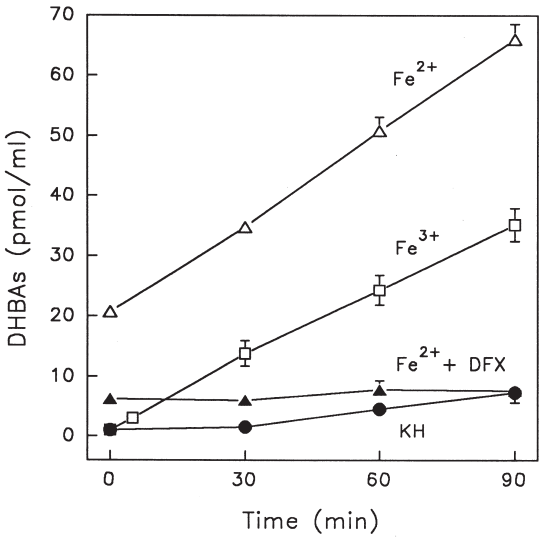

Figure 4. Formation of 2,5- plus 2,3-dihydroxybenzoic acids (DHBAs) in the media containing ascorbate.

Formation of DHBAs upon addition of ascorbate to Krebs-Henseleit buffer (KH) alone (˘) or containing: 10 μM Fe^{3+} (£), 10 μM Fe^{2+} (Δ), and 10 μM Fe^{2+} + 50 μM desferrioxamine (s) was measured. The values represent the mean ± S.E.M. of 5 determinations.

Ascorbate initiated ·OH production not only in the media containing Fe^{3+} but also in those containing Cu^{2+} (Fig. 5). This ability was shared also by reduced glutathione, cysteine and homocysteine.

The efficiency of Fe^{2+}-O_2 chemistry* vs *Fe^{2+}-driven Fenton reaction to generate ·OH. Addition of H_2O_2 (0–35 μM) to KH resulted in a small increase in DHBAs production (from 0.96 pmol/ml at zero H_2O_2 to 1.9 pmol/ml at 35 μM H_2O_2). Addition of 1 μM, 5 μM or 10 μM Fe^{2+} to a range of H_2O_2 concentrations resulted in an H_2O_2-concentration dependent rise in ·OH formation (Fig. 6). The curves for 1 μM, 5 μM and 10 μM Fe^{2+} saturated at about 1 μM, 5 μM and 10 μM H_2O_2, respectively, suggesting that the H_2O_2 : Fe^{2+} concentration ratio of approximately one was optimal for the reaction between Fe^{2+} and

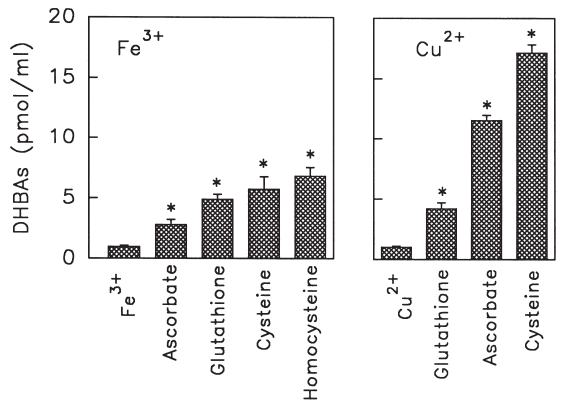

Figure 5. Comparison of the enhancing effects of ascorbate, reduced glutathione, cysteine and homocysteine on 2,5- plus 2,3-dihydroxybenzoic acids (DHBAs) formation in the reaction mixture containing either 10 μM Fe^{3+} (left) or 10 μM Cu^{2+} (right).

The experiments with Fe^{3+} were carried out at pH 7.4 and the incubation time was 5 min. In the experiments with Cu^{2+}, pH was 6.6 and the incubation time was 30 min. Ascorbate, reduced glutathione (GSH), cysteine, and homocysteine were 200 μM. The values represent the mean ± S.E.M. of 5 determinations. $^*P < 0.05$ *vs* Fe^{3+} or Cu^{2+} alone, respectively.

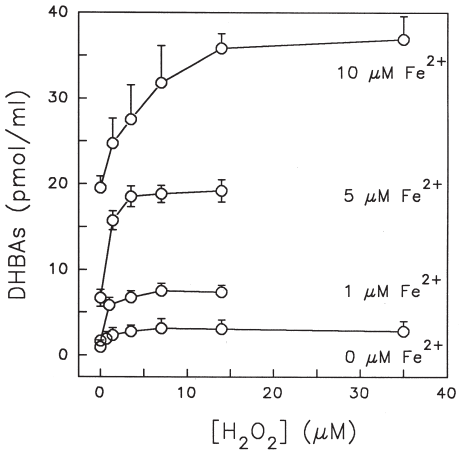

Figure 6. Efficiency of the autoxidation reaction in comparison with the Fenton reaction.

Fe^{2+}-mediated formation of 2,5- plus 2,3-dihydroxybenzoic acids (DHBAs) in media containing H_2O_2 was measured. The indicated concentration of Fe^{2+} was incubated for 1 min in oxygenated Krebs-Henseleit buffer containing the indicated concentrations of H_2O_2. The measurements obtained at zero H_2O_2 represent DHBAs production mediated solely in the autoxidation reaction of the indicated Fe^{2+} concentration. The values represent the mean ± S.E.M. of 5 determinations.

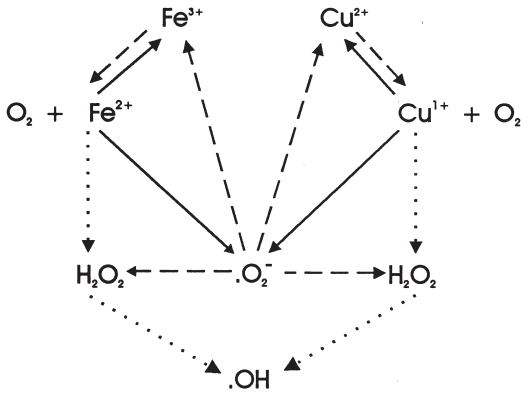

Figure 7. Hypothetical reaction pathway initiated by the addition of Fe^{2+} or Cu^{+} to the oxygenated medium contaminated with traces of Fe^{3+} and Cu^{2+}.

The reaction starts with the autoxidation of the metal (—). Some part of the originating $O_2^{\cdot-}$ dismutates to H_2O_2 and some interacts with Fe^{3+} and Cu^{2+} (contaminating and/or newly formed), to recover their reduced forms (- - -). The probability that $O_2^{\cdot-}$ meets Fe^{3+} and/or Cu^{2+} and the metals undergo the redox cycling, increases (up to a certain optimum) with the Fe^{3+} and Cu^{2+} concentration. Finally, the metal-driven Fenton reaction follows, resulting in $^{\cdot}OH$ liberation (· · ·). The scheme ignores the mechanism of the exceptional role of the metal redox cycling in the $^{\cdot}OH$ generation process. See text for more explanation and the discussion.

H_2O_2 (the Fenton reaction) to produce $^{\cdot}OH$. Of note, for each Fe^{2+} concentration used the $^{\cdot}OH$ yield at each optimal H_2O_2 concentration was a sum of the $^{\cdot}OH$ deriving from the Fe^{2+}-O_2 reaction and that from the maximally activated Fenton reaction. It is evident from Fig. 6 that for each Fe^{2+} concentration, the $^{\cdot}OH$ yield approximately doubled when the H_2O_2 concentration was raised from zero to the optimal level. Thus, at the optimal H_2O_2 : Fe^{2+} ratio, only 50% of the total $^{\cdot}OH$ yield is attributable to the Fenton reaction.

DISCUSSION

Experimental conditions of the study

$^{\cdot}OH$ generation was studied here in KH because it is routinely used in physiological stud-

ies, and because phosphate serves as a part of the buffering capacity, both of KH and of the cell. Metal autoxidation is a slow process unless the metal is liganded to an appropriate chelator (e.g., EDTA). Because phosphate buffer has been shown to catalyse Fe^{2+} autoxidation [6, 8], it was important to see if the reaction is possible also in the presence of the low phosphate concentration present in KH and without the need of a non-physiological chelator. Indeed, we report that Fe^{2+}-mediated DHBAs yield was 4.5-fold greater in KH compared with that in phosphate-free KH. Thus, although salicylate can chelate iron [6], and bicarbonate can promote ·OH generation from Fe^{2+} and O_2 [8], the chemistry of our system seems to be mediated predominantly by the low, physiological concentration of phosphate. Of note, it has been demonstrated that *in vivo* infusion of Fe^{2+} through a dialysis probe into rat myocardium results in an increased ·OH generation in the myocardial extracellular fluid [11], suggesting that ·OH generation mediated by the Fe^{2+}-O_2 chemistry is possible also in biological systems. To avoid confounding the assessment of ·OH by radicals originating from glucose oxidation [12], the sugar was omitted from KH. Given all these conditions, the detection of salicylate hydroxylation, employed in this study, provided a specific method for quantitative analysis of only the metal-mediated and predominantly phosphate promoted ·OH generation [13].

Furthermore, micromolar concentrations of iron and copper ions were used. These concentrations seem to be physiologically relevant, for instance, in the context of myocardial ischaemia/reperfusion. Ischaemia has been demonstrated to redistribute sequestrated intracellular iron into the low molecular weight pool [14, 15] and in KH perfused isolated rat heart this pool was estimated to increase from 2 μM up to 54 μM [15]. In addition, submicromolar and micromolar concentrations of copper and iron, respectively, have been shown to be released from the perfused rat heart upon reperfusion [16,17]. Of note, in our previous measurements, using the same salicylate method as employed here, the post-ischemic DHBAs outflow in KH perfused isolated guinea pig hearts amounted to 4–7 pmol/ml [18]. As evident from Fig. 2, this amount is comparable to the DHBAs production mediated *in vitro* by about 5 μM Fe^{2+}, a concentration encountered in the isolated ischaemic/reperfused rat heart [16, 17].

The mechanism of Fe^{2+}- and Cu^{+}-mediated ·OH generation

The exact mechanism of the ·OH generation is not apparent from this study, although some of its elements may be delineated. We demonstrate that the process in which iron and copper ions promote ·OH formation is critically dependent on the availability of O_2 (Fig. 1), is attenuated by SOD and catalase (Table 1), and is dependent on the redox state of the metal. The latter is evidenced by the fact that Fe^{3+} and Cu^{2+} ions were able to promote ·OH formation only in the presence of various reducing agents (Figs. 2 and 5), indicating that Fe^{2+} and Cu^{+} rather than Fe^{3+} and Cu^{2+} initiate ·OH production. Although only ·OH was measured here, these results implicate O_2 as the oxidant, with the formation of $O_2^{\cdot-}$ and H_2O_2 being critical for ·OH formation in the media containing the reduced forms of the transition metals. This, in turn, is consistent with the idea that the metal autoxidation (Eqn. 4) [6–9] is a part of the ·OH generation mechanism (see later, discussion on the SOD effect).

Furthermore, three lines of evidence illustrate the fact that, in analogy to the Fenton chemistry [19, 20], also the Fe^{2+}-O_2 and Cu^{+}-O_2 chemistry is critically dependent on the Fe^{3+} : Fe^{2+}, Cu^{2+} : Fe^{2+} and Fe^{3+} : Cu^{+} concentration ratios, suggesting that metal redox cycling is a part of the ·OH generation mechanism. First, the Fe^{2+}- and Cu^{+}-mediated ·OH generation was inhibited by the specific Fe^{3+} chelator DFX, indicating that the Fe^{2+}- and

Cu^{+}-mediated reaction was dependent on the contaminating Fe^{3+}. Of note, 10 μM and 50 μM DFX appeared similarly potent in inhibiting the Fe^{2+}-mediated $^{\cdot}OH$ generation, indicating that maximally effective DFX concentrations were studied. In addition, the nonspecific Fe^{3+}/Fe^{2+} chelator DTPA appeared to be significantly more effective than DFX in inhibiting the Fe^{2+}-mediated $^{\cdot}OH$ yield (Table 1). From these we conclude that, indeed, the effect of DFX was specifically related to the chelation of Fe^{3+}, but not of Fe^{2+}, and was not related to oxygen free radical scavanging properties of DFX [21]. Second, the addition of Fe^{3+} or Cu^{2+} to the reaction mixture was found to augment the Fe^{2+}-mediated $^{\cdot}OH$ generation (Fig. 3). Third, when studied in the standard KH, the Fe^{2+}-mediated reaction appeared to die out within 30 s (Fig. 1). However, in the presence of an excess of the reductant (ascorbate), the reaction continued for at least 90 min, an effect abolished by DFX (Fig. 4). These results suggest that while the reductant maintains the reaction by enabling continuous redox cycling of iron ($Fe^{2+} \rightarrow Fe^{3+} \rightarrow Fe^{2+}$), Fe^{3+} chelation prevents iron ions from entering into the redox cycles necessary for $^{\cdot}OH$ formation.

To combine the discussed observations into one mechanism we propose a simplified scheme in which at least four individual reactions (Eqns. 4, 1, 3 plus the metal redox cycling reaction), interacting with one another, as speculated in Fig. 7, are involved in the Fe^{2+}- and Cu^{+}-promoted $^{\cdot}OH$ generation. Of note, the scheme also predicts that, in agreement with our results, in media contaminated with Fe^{3+} and Cu^{2+}, the reaction mediated by Fe^{2+} would initiate simultaneous redox cycling of copper ions, and *vice versa*. Consequently, the amount of measured $^{\cdot}OH$ would be always the sum of $^{\cdot}OH$ generated processes driven by iron and copper ions (Fig. 7). The limitation of the scheme is, however, that it ignores the exact (additional?) mechanism in which $^{\cdot}OH$ production is dependent on the $Fe^{3+} : Fe^{2+}$, $Cu^{2+} : Fe^{2+}$ and $Fe^{3+} : Cu^{+}$ concentration ratio, the mechanism which is not known at the moment [1, 19]. Actually, several aspects of the Fenton chemistry are currently ill-understood and the main unanswered question concerns the role of intermediate oxidants like ferryl and perferryl species and metal-dioxygen complexes [3, 4, 22]. In this context, our results imply that the mechanism of the Fe^{2+}-mediated $^{\cdot}OH$ production consists of at least two components. The majority of the $^{\cdot}OH$ seems to be produced in the mechanism secondary to the Fe^{3+}/Fe^{2+} redox cycling, as evidenced by the approximately 80% inhibition of the $^{\cdot}OH$ production by DFX (Table 1). The other component would be specifically Fe^{2+}-dependent (possibly Fe^{2+} autoxidation-dependent), the conclusion supported by the fact that $^{\cdot}OH$ yield from the Fe^{2+}-mediated reaction was significantly more inhibited by DTPA than by DFX (used in the concentration producing maximum Fe^{3+}-chelation) (Table 1). Our observation that SOD attenuated $^{\cdot}OH$ yield from Fe^{2+}-mediated reaction would be in line with the dominating role of Fe^{3+}:Fe^{2+} and/or the metal redox cycling in the $^{\cdot}OH$ generation mechanism. SOD, by promoting $O_2^{\cdot-}$ dismutation, is expected to increase H_2O_2 production at the expense of the decreasing $O_2^{\cdot-}$ concentration. This would have two opposing consequences for $^{\cdot}OH$ generation. The rise in H_2O_2 would promote the liberation of $^{\cdot}OH$. However, the fall in $O_2^{\cdot-}$ would prevent the metal redox cycling ($O_2^{\cdot-}$-induced reduction of the metal is decreased) and the resulting $^{\cdot}OH$ generation. We speculate that this second consequence of SOD treatment prevailed in our experimental conditions.

The efficiency of Fe^{2+}-O_2 vs Fe^{2+}-driven Fenton reaction in generating $^{\cdot}OH$

As proposed in Fig. 7, Fe^{2+} and Cu^{+}, added to the medium like KH, may play a dual role: they generate $O_2^{\cdot-}$, presumably in the autoxidation reaction, and liberate $^{\cdot}OH$ from H_2O_2 in the Fenton-type reaction. Our data indicate that in the systems containing an additional

source of $O_2^{\cdot-}$ and/or H_2O_2, as it may be the case in biological systems, the efficiency of the Fe^{2+}-O_2 chemistry to generate $^{\cdot}OH$ may be greater than, or at best, equal to that of the Fe^{2+}-driven Fenton reaction, depending on the actual H_2O_2 : Fe^{2+} concentration ratio (Fig. 6).

The biological relevance of the results

Extrapolated to biological systems, these data suggest that tissues exposed to an increased concentration of iron and/or copper (e.g., liberated from internal stores) may be prone to oxidative damage related to the metal ion-O_2-mediated free radical production. This might be indeed so, because transition metals, when liberated from intracellular stores, are probably present in reduced forms [2, 3].

If it is taken for granted that increased pool of low molecular mass iron and copper is present in ischemic tissues [14, 15, 23], it becomes apparent that reperfusion, which induces tissue injury in a mechanism involving $^{\cdot}OH$ [24], creates particularly favourable conditions for the metal ion-O_2 reaction to occur. This is because: (i) this reaction is fast enough to account for the reperfusion-induced production of free radicals (Fig. 1); (ii) O_2, catalytic metals, and their reductants (including enzymatically produced $O_2^{\cdot-}$) are abundant, and (iii) intracellular pH rapidly changes in the alkaline direction [25] in the reperfused tissue, the condition reported here (Fig. 3) and by others [26] to facilitate metal ion-O_2 chemistry.

In view of the production of $^{\cdot}OH$ caused by homocysteine (Fig. 5), there is evidence to link high plasma levels of homocysteine to atherothrombosis in humans [27]. There is also evidence that the pro-oxidative properties of ascorbate [28, 29] (Figs. 4 and 5] are of a biological relevance [29, 30].

It must be realized, however, that the metal ion-O_2 chemistry was studied here in a medium devoid of proteins and containing a relatively high O_2 concentration (pO_2 about 600 mmHg *vs.* 100 mmHg in the arterial blood), which may limit the biological relevance of our results, e.g., to organs perfused with buffered saline solutions only.

Altogether, our results provide further evidence that: (i) Fe^{2+}-O_2 and Cu^{+}-O_2 chemistry mediates $^{\cdot}OH$ production, also in KH, and possibly in a mechanism initiated by the metal ion autoxidation; (ii) the reactions mediated by Fe^{2+} and Cu^{+} interact with each other; (iii) the efficiency of the Fe^{2+}-O_2 system to generate $^{\cdot}OH$ in KH is greater than or equal to that of the Fe^{2+}-driven Fenton reaction. We speculate that, at least in some biological systems, iron and copper ions may constitute an efficient source of the reactive oxygen species, without the requirement for pre-existing enzymatically generated $O_2^{\cdot-}$ and H_2O_2.

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